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Article

GLYPHOSATE APPLIED AT THE EARLY REPRODUCTIVE STAGE IMPAIRS SEED PRODUCTION OF GLYPHOSATE-RESISTANT HAIRY FLEABANE

Glyphosate Aplicado no Início do Estádio Reprodutivo Inviabiliza a Produção de Sementes de Buva Resistente ao Glyphosate

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ABSTRACT - Glyphosate-resistant hairy fleabane [*Conyza bonariensis* (L.) Cronq.] is one of the most important weeds in the world. Among the factors that make this weed species widely distributed in the most diverse environments is the high seed production capacity and dispersal. Hairy fleabane plants not controlled by herbicide application regrowth and overcome crop canopy, use environmental resources, interfere with crops, and complete their life cycle by producing thousands of seeds and replenishing the seed bank. Management strategies that reduce production and viability of hairy fleabane seeds can be adopted within the integrated management to reduce the seed bank and prevent further infestations. In this way, experiments were carried out in a greenhouse and laboratory of seed analysis to evaluate the effect of glyphosate (1,480 g a.e. ha^{-1}) on the production and viability of glyphosate-resistant hairy fleabane seeds when applied at the vegetative and reproductive stages. Seed production was reduced by 68.4 and 100% when glyphosate was applied on hairy fleabane plants at the vegetative and early reproductive stages, respectively, regarding to the control. The viability of hairy fleabane seeds was not influenced by treatments at the evaluated stages. However, glyphosate treatment reduced the hairy fleabane seed production when applied at the vegetative stage . Hairy fleabane seed production is not feasible when glyphosate is applied at the early reproductive stage.

Keywords: *Conyza bonariensis*, resistance, integrated weed management, prevention, soil seed bank.

*RESUMO - A buva [**Conyza bonariensis** (L.) Cronq.] resistente ao glyphosate é uma das principais plantas daninhas do mundo. Um dos fatores que fazem com que esta planta esteja amplamente distribuída nos mais diversos ambientes é sua alta capacidade de produção e dispersão de sementes. Plantas de buva não controladas pela aplicação de herbicidas rebrotam e sobressaem-se ao dossel da cultura, se beneficiam dos recursos do ambiente, interferem com as culturas, completam seu ciclo produzindo milhares de sementes e realimentando o banco de sementes. Estratégias de manejo que reduzam a produção e viabilidade de sementes de buva podem ser adotadas dentro do manejo integrado, a fim de o banco de sementes e prevenir futuras infestações. Foram realizados experimentos em casa de vegetação e em laboratório de análise de sementes com o objetivo de avaliar o efeito da aplicação de glyphosate nos estádios vegetativo e reprodutivo na produção e viabilidade de sementes de buva resistente ao glyphosate (1.480 g e.a. ha^{-1}). Os resultados demonstram que a produção de sementes reduziu em 68,4% e 100% quando o glyphosate foi aplicado em plantas de buva nos estádios vegetativo e início do reprodutivo, respectivamente, em relação a plantas não tratadas. A viabilidade das*

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sementes de buva não foi influenciada pelos tratamentos nos estádios avaliados. Entretanto, a aplicação do glyphosate no estádio vegetativo da buva reduz a produção de sementes. A produção de sementes de buva é inviabilizada quando o glyphosate é aplicado no início do estádio reprodutivo.

Palavras-chave: *Conyza bonariensis*, resistência, manejo integrado de plantas daninhas, prevenção, banco de sementes do solo.

INTRODUCTION

Weeds of the genus *Conyza* are among the most problematic, harmful, invasive, and widely distributed in agriculture (Bajwa et al., 2016). Hairy fleabane (*C. bonariensis*) belongs to the botanical family Asteraceae, is native to the Americas, and currently has a cosmopolitan distribution (Shrestha et al., 2014; Bajwa et al., 2016). It has an annual cycle plant that presents an incomplete autogamy with a cross-pollination of up to 15%, being reproduced by seeds (Zelaya et al., 2007; Stewart et al., 2009). A single hairy fleabane plant can produce more than 200,000 seeds (Bhowmik and Bekech, 1993). The hairy fleabane seeds are small (pappus + cypsela from 4 to 4.7 mm), very light and with morphological structures that facilitate dispersion, which can surpass the distance of 100 meters (99% of seeds up to 100 m and 1% up to 500 m) (Urdampilleta et al., 2005; Dauer et al., 2007). These characteristics, coupled with resistance to glyphosate and no-tillage system, are factors that contributed to the large expansion and adaptation of this weed in different environments (Bajwa et al., 2016).

Hairy fleabane seeds are easily dispersed, mainly by wind, favoring its presence in extensive agricultural areas in Brazil (Vargas et al., 2016). In general, hairy fleabane plants develop during periods that coincide with soybean cultivation, and the interference caused by one plant per square meter can cause losses in soybean yield up to 36% (Trezzi et al., 2015). Also, the control cost of glyphosate-resistant hairy fleabane may be up to five times higher when compared to the control of susceptible plants of the same species (Vargas et al., 2016).

The management of hairy fleabane in agricultural systems has been carried out intensively through herbicide application, mainly glyphosate. Glyphosate is widely used because of its high effectiveness and low cost, being low harmful to the environment when compared to other herbicides (Peng et al., 2010). However, the intense and large-scale use of glyphosate has been an essential factor in the evolution of resistance, making herbicide application inefficient (Baucom and Holt, 2009).

In recent years, failures in hairy fleabane control have been attributed to problems with resistance to herbicides and its incorrect application (Vargas et al., 2016). Plants not controlled after herbicide application regrowth and overcome crop canopy, causing interference and consumption of environmental resources, completing their life cycle in time similar to the crop. Upon completion of the cycle, the production of hairy fleabane seeds replenishes the seed bank, favoring future infestations (Bajwa et al., 2016). The replenishment of the soil seed bank directly involves weed management, as it favors the occurrence of high plant densities in future generations, interfering with and causing crop losses. Moreover, high weed densities favor an increase in the frequency of occurrence and selection of biotypes containing alleles responsible for conferring resistance to herbicides within each population (Radosevich et al., 2007). Therefore, management strategies that reduce the production and viability of hairy fleabane seeds and consequent reduction of soil seed bank can be useful for integrated management of this important weed within the productive system.

This study hypothesized that glyphosate application at different growth stages of hairy fleabane interferes with the production and viability of seeds. In order to test these hypotheses, experiments were carried out to evaluate the effect of glyphosate application on the production and viability of glyphosate-resistant hairy fleabane seeds treated at the vegetative and reproductive stages.

MATERIAL AND METHODS

The experiments were conducted in a greenhouse and laboratory of seeds at Universidade Federal de Pelotas (UFPel), from August 2017 to March 2018. The experiments were conducted

using a single hairy fleabane biotype (B11R – originated from Pelotas, RS, 32°04'05.91" S and 52°52'59.14" W) (Piasecki et al. 2019a; Piasecki et al. 2019b). The hairy fleabane biotype used has a high glyphosate resistance factor (RF 18.4 – GR_{50}, growth reduction), which was determined in two experiments evaluated at 28 days after herbicide application in hairy fleabane at 60 days after emergence (Piasecki et al. 2019a; Piasecki et al. 2019b). The species *C. bonariensis* was used. The identification of the species was performed by genotyping using SSR (simple sequence repeats) molecular markers, according to Abercrombie et al. (2009) and Marochio et al. (2017) (Piasecki et al. 2019a; Piasecki et al. 2019b).

Greenhouse experiment and seed collection

The greenhouse experiment was conducted in a completely randomized design with four replicates. Treatments consisted of two glyphosate doses (0 and 1,480 g a.e. ha^{-1}; Roundup Original DI 370 g a.e. L^{-1}; Monsanto) applied on hairy fleabane plants at the vegetative stage (30 days before the reproductive stage – plants at 1.10 m in height) and at the early reproductive stage (beginning of reproductive structure formation; plants at 1.40 m in height) (Figure 1).

(A) Plants at the early reproductive stage (detail: formation of reproductive structures); (B) Plants at the vegetative stage.

Figure 1 - Growth stage of hairy fleabane plants on the day of glyphosate application.

Seeds that originated hairy fleabane plants used in the experiment came from the same mother plant (B11R), which did not receive glyphosate application. Sowings were carried out with a 30-day interval (vegetative stage on September 1, 2017; reproductive stage on August 2, 2017), being cultivated under the same conditions. In a controlled environment, hairy fleabane seeds were sown in plastic trays containing soil and substrate (Mac plant – Mec Prec, Brazil) previously sterilized in an autoclave (1 atm – 132 °C for 60 min) in the proportion of 3:1 and irrigated daily at 25/15 °C (± 4 °C) day/night, with a 12 hour photoperiod (light – 24,275 lumens m^{-2}; color temperature ≥ 6,500 Kelvin). At 30 days after emergence (DAE) (seeds emerged within five days after sowing), plants of each stage were individually transplanted into 5 L pots containing soil and substrate, as previously described. After transplanting, plants were maintained in the same greenhouse, with temperature and natural photoperiod for the region of Pelotas, RS.

When the plants that were first sown reached the reproductive stage (beginning of the formation of reproductive structure) (Figure 1), the treatment with glyphosate was carried out in plants of the two stages. On the application day, plants at the vegetative stage were shorter when compared to those at more advanced stages, with no formation of reproductive structures (Figure 1). Four plants of each stage received the application of glyphosate, and four other plants per treatment were not treated (control treatment). The application was conducted with a CO_2-pressurized backpack sprayer with a volume of 150 L ha^{-1} and flat fan spray nozzles 110-02 (XR Teejet). Seeds of each plant at full physiological maturity, naturally detached from the mother plant, were collected daily and individually packaged in properly identified paper bags. Collections were performed manually by introducing the branches containing reproductive structures into paper bags in the horizontal position and then performing a gentle shaking to avoid damaging the plant. During the collection period, paper bags containing the previously collected seeds remained in the same greenhouse until the end of the experiment. After the end of the experiment, the shoot and roots of plants were individually separated and dried in an air circulation oven at 60 °C until constant weight, being weighed on a precision scale and determined the total plant dry matter.

Analysis of hairy fleabane seeds in the laboratory

After the greenhouse experiment was finished, seeds packed in paper bags were placed in a refrigerator (~4 °C) for fourteen days to overcome dormancy and standardize germination (personal observations). After that period, seeds of each sample were homogenized and weighed (total seed weight), being determined the one thousand-seed weight (TSW) and estimated the number of seeds produced per plant. The total sample weight was determined on a precision scale with five decimal places, in a closed chamber. TSW was obtained by random counting (using a 40X magnifier and tweezers) 1,000 hairy fleabane seeds per replication (4 x 250 at each replication), followed by weight determination on a precision scale. The number of seeds produced per plant was estimated by a simple rule of three from the total seed weight per plant and TSW.

The germination test was carried out to evaluate the physiological quality of hairy fleabane seeds, in which 100 seeds were randomly selected per repetition using a 40X magnifier and tweezers. Subsequently, seeds were distributed on two sheets of blotting paper and packed in a gearbox (12 × 12 cm – 100 cells each). The sheets were previously moistened with an amount of distilled water equivalent to three times their dry weight. The experiment was conducted under a 24 hour light photoperiod at 20 °C in a BOD-type germination chamber (Vidal et al., 2007).

The percentage of germination was determined at 14 days after sowing, in which the number of normal seedlings was evaluated. Together with the germination test, the first germination count was carried out at seven days after sowing to determine seed vigor (Vivian et al., 2008). After evaluating the number of normal seedlings of the germination test, the number of empty, dead, and dormant seeds were counted, and the results expressed in percentage.

The viability of non-germinated seeds was evaluated at the end of the germination test by the tetrazolium test using the salt 2,3,5-triphenyl tetrazolium chloride at 1.0%, being considered viable the seeds with pink or carmine coloring in the whole seed (Brasil, 2009) and characterized as dormant. For this, seeds were placed in a tetrazolium solution inside a transparent glass container closed with aluminum foil to prevent light from entering during 24 hours at 30 °C

(Brasil, 2009). The percentages of viable and non-viable seeds were calculated based on the number of seeds that were dormant in the germination test.

The data were submitted to analysis of variance (ANOVA) by the F-test ($p \leq 0.05$). When the F-test was significant, means were compared by the Student t-test ($p \leq 0.05$). Comparisons were performed between the mean results obtained within each stage in the treatments with and without glyphosate treatment. Descriptive statistics were used to compare the plant's stages.

RESULTS AND DISCUSSION

The results showed a difference for the total weight of seeds produced by hairy fleabane plants treated with glyphosate at the vegetative stage in relation to the control treatment. At this stage, there was no difference for TSW, but the number of seeds was 68.4% lower in treated plants when compared to the control, which produced an estimated number of 121.5 thousand of seeds (Table 1). There was no seed production when hairy fleabane plants were treated with glyphosate at the early reproductive stage. Thus, the total weight, TSW, and the number of seeds were 100% lower when compared to the control, which produced an estimated number of 75.6 thousand of seeds (Table 1 and Figure 2).

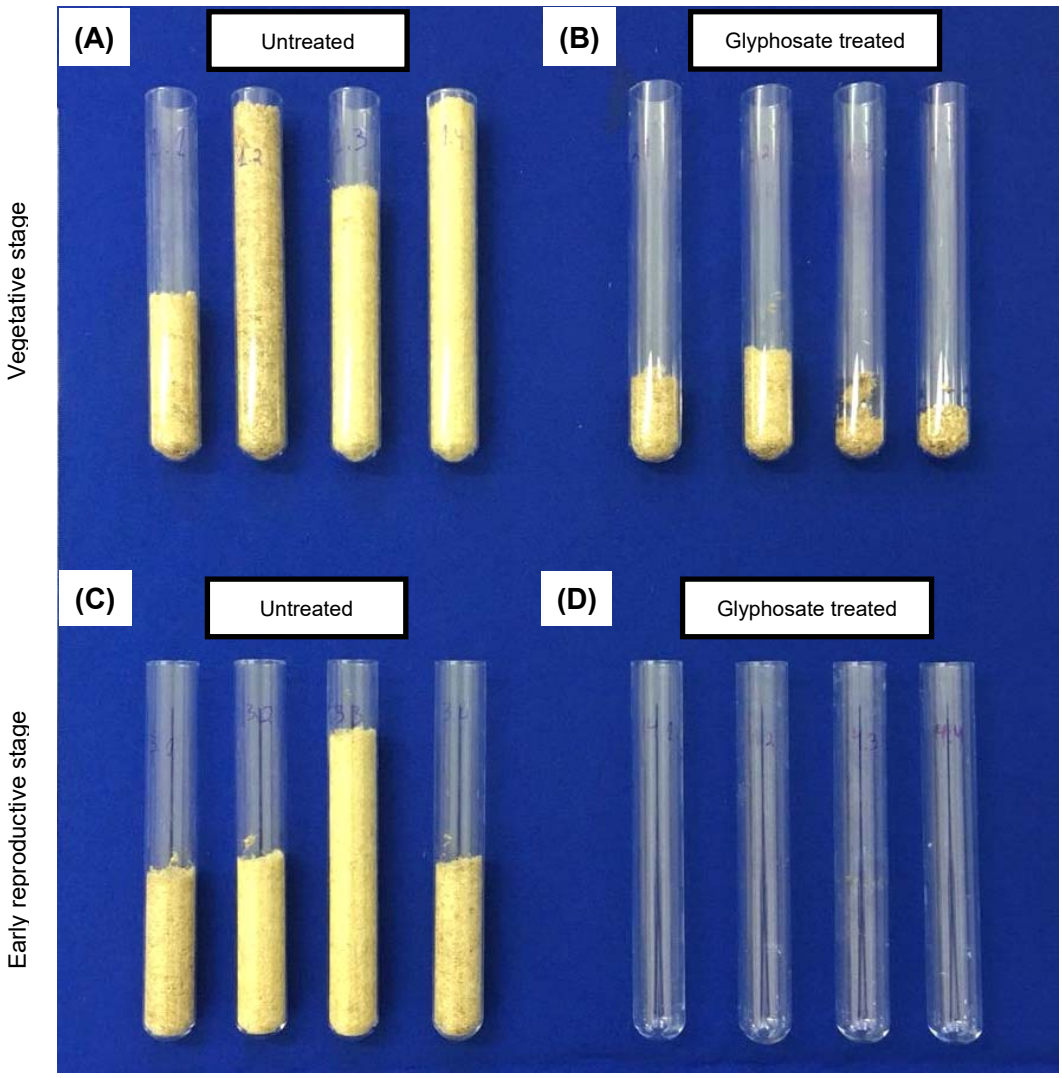
Table 1 - Total seed weight per plant, one thousand-seed weight (TSW), and the estimated number of seeds of hairy fleabane plants followed by glyphosate treatment at different growth stages

Treatment	One thousand-seed weight (mg)[(1)]	TSW (mg)[(2)]	Number of seeds[(3)]
Vegetative stage			
Without glyphosate	4,845	40.5	121,482
With glyphosate	1,092	33.9	38,365
p-value	0.00406*	0.585^{NS}	0.03*
Reproductive stage			
Without glyphosate	3,542	49.7	76,408
With glyphosate	0	0	0
p-value	0.014*	0.0019*	0.044*

[(1)] Mean of the total seed weight per hairy fleabane plant; [(2)] One thousand-seed weight; [(3)] Estimated mean number of seeds per hairy fleabane plant; * Significant by the t-test for samples with two-tailed distribution and unequal variation of two samples (heteroscedastic) with confidence of $p \leq 0.05$; [NS] Not significant by the t-test at a confidence of $p \leq 0.05$.

From five days after glyphosate application, plants treated at both stages showed symptoms of marked chlorosis at the apexes. Inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme by glyphosate reduces the production of the aromatic amino acids phenylalanine, tyrosine, and tryptophan, the latter the precursor of the vegetable hormone indole-acetic acid (IAA). The reduction in IAA concentration in apical meristems of plants leads to loss of dominance, followed by epinasty (Cobb and Reade, 2010). Also, glyphosate application at the early reproductive period may affect IAA levels in seeds and inhibit germination and emergence of seedlings (Clay et al., 2000). The effects of chlorosis occur as a metabolic consequence of the inhibition of the enzyme δ-aminolaevulinic acid (δ-ALA) synthetase, which acts on the biosynthesis of chlorophylls and cytochromes (Cobb and Reade, 2010).

Plants treated with glyphosate at early reproductive stage, in addition to chlorosis, presented epinasty and formation of a type of ring of 0.5 to 1.5 cm in length positioned about ten centimeters below the base of each reproductive structure (Figure 3). Initially, this ringing presented a water-soaking appearance and, ten days after treatment, evolved to necrosis from that point to the apex, resulting in the unfeasibility of reproductive structures (Figure 3). In this situation, and despite hairy fleabane plants are resistant to glyphosate, they did not produce seeds and died at 40 days after treatment and about 30 days before control plants, shortening their life-cycle. It indicates that plants did not have energy stored enough to overcome the disturbances caused by the action of glyphosate and produce new branches or reproductive structures. This result indicate that glyphosate-resistant hairy fleabane plants are more sensitive to herbicide applications at the reproductive stage.



(A) Plant without glyphosate application; (B) Plant with glyphosate application (1,480 g a.e. ha^{-1}) at the vegetative stage; (C) Plant without glyphosate application; (D) Plant with glyphosate application (1,480 g a.e. ha^{-1}) at the early reproductive stage. Each test tube represents the total seeds produced by a hairy fleabane plant.

Figure 2 - Volume of hairy fleabane seeds produced per plant as a function of treatments.

Plants invest a large part of the energy accumulated during the vegetative period in reproduction (Taiz et al., 2017). Thus, when differentiating reproductive structures, plants are more vulnerable to the glyphosate' action, mainly when applied at the early reproductive stage (Walker and Oliver, 2008). It is worth noting that during seed maturation, any type of stress that restricts plant metabolism during its growth and development can drastically affect photoassimilate production and decrease the amount of metabolic products that would be sent to seeds during their formation (Barnabás et al., 2008). Stress conditions favors the production of seeds it favors the production of seeds with low reserves and reduced accumulation of important germination proteins (Taiz et al., 2017), making them of low physiological quality.

Physiological quality results showed that hairy fleabane seeds were more affected when plants were treated with glyphosate at the early reproductive stage because of the non-seed production. No difference was observed when the application was performed at the vegetative stage (Table 2). Table 2 also shows a low quality and viability of hairy fleabane seeds, even in the controls, in relation to the 100% potential. Although each plant produced thousands of seeds, only 14 and 63% (mean 38.5%) germinated in the control of the vegetative and reproductive stages, respectively. Costa et al. (2018) observed that the viability of hairy fleabane seeds from glyphosate-resistant and -sensitive biotypes was lower than 50%. The germination of hairy

(A and B) Epinasty, ringing, and water-soaking at the base of the reproductive structure, with evolution to necrosis; (C) Onset of necrosis of reproductive structures (15 DAT); (D) Total necrosis of the reproductive structures (28 DAT).

Figure 3 - Symptoms caused by glyphosate' action at the early reproductive stage of hairy fleabane plants.

Table 2 - Germination, first germination count (FGC), abnormal seedlings, dead, dormant, viability by tetrazolium test, and percentage of empty hairy fleabane seeds submitted to glyphosate application at different phenological stages

Stage	Glyphosate application	Germination	FGC(1)	Abnormal	Dead	Empty	Dormant	Viable(2)	Unviable(2)
		(%)							
Vegetative	Without	14	12	1	5	66	14	7	93
	With	1	0	0	1	95	3	0	100
	p-value	0.34^{NS}	0.34^{NS}	0.21^{NS}	0.12^{NS}	0.11^{NS}	0.15^{NS}	0.39^{NS}	0.23^{NS}
Early reproductive	Without	63	38	4	7	13	13	10	90
	With	0	0	0	0	0	0	0	0
	p-value	0.0006*	0.002*	0.077^{NS}	0.024*	0.0007*	0.021*	0.194^{NS}	0.0007*

(1) FGC: first germination count (vigor) (7 DAS); (2) Percentage in relation to dormant seeds; * Significant by the t-test for samples with two-tailed distribution and unequal variation of two samples (heteroscedastic) with confidence of p≤0.05; ^{NS} Not significant by the t-test at a confidence of p≤0.05.

fleabane seeds varies according to environmental conditions (light, temperature, salinity, and sowing depth), varying from 18 to 32% under ideal conditions (Nandula et al., 2006; Wu et al., 2007; Davis et al., 2008).

The total dry matter (shoot and root) of hairy fleabane plants was not influenced by glyphosate application nor by growth stages (Table 3). It indicates that, although a difference of 30 days was observed in plant emergence (vegetative × reproductive), the dry matter production was similar between them. Thus, the difference in seed production and viability is mainly due to the differential effect of glyphosate on the different plant stages at the application time and not to differences in the cycle and/or lack of time for photoassimilate production.

Herbicide application at advanced weed stages aiming reducing seed production and viability has been reported as an effective alternative. Clay and Griffin (2000) evaluated seed production and the emergence of *Xanthium strumarium*, *Sesbania exaltata*, and *Senna obtusifolia* after glyphosate application at three stages: the beginning of seed filling, half of the seed filling, and physiological maturation of seeds. The authors concluded that when glyphosate was applied at the beginning of filling, there was a reduction in seed production and emergence of those species in relation to the control, with values of 69 and 97% (*X. strumarium*), 86 and 94% (*S. exaltata*), and

83 and 63% (*S. obtusifolia*), respectively. Isaacs et al. (1989) found that glyphosate application before fruit production in *Cassia obtusifolia* reduced the number of seeds by more than 90% but did not alter the emergence. Bennett and Shaw (2000) studied the effect of mixtures between glyphosate plus sodium chlorate and paraquat plus sodium chlorate applied in soybean pre-harvest and concluded that these applications reduced seed production and germination of *S. obtusifolia*, *S. exaltata*, and *Ipomoea lacunosa*. Another study carried out by Biniak and Aldrich (1986), evaluated seed production and viability of *Abutilon theophrasti* and *Setaria faberi* after the application of the herbicides chlorflurenol, chlorsulfuron, and glyphosate at the beginning of flowering. The authors concluded that glyphosate was the most efficient among studied herbicides, reducing seed production and emergence by 99 and 50% (*A. theophrasti*) and 96 and 95% (*S. faberi*), respectively.

Table 3 - Total dry matter (shoot and roots) of hairy fleabane plants submitted to glyphosate application at different growth stages

Treatment	Total plant dry matter (g)
Vegetative stage	
Without glyphosate	102.3
With glyphosate	106.3
p-value	0.69^{NS}
Early reproductive stage	
Without glyphosate	125.0
With glyphosate	128.1
p-value	0.75^{NS}

^{NS} Not significant by the F test at $p \leq 0.05$.

In agricultural systems, the production of weed seeds plays a fundamental role in the reproduction and succession of species. Weed seeds may remain viable in the soil for more than ten years, leading to emergence flows and making management difficult (Radosevich et al., 2007). The longevity of hairy fleabane seeds in the soil is approximately three years (Wu et al., 2007). However, the high number of seeds produced by hairy fleabane plants favors the replenishment of soil seed bank.

Thus, glyphosate application at the early reproductive stage of hairy fleabane can be used as an efficient strategy to prevent and reduce seed production and replenish soil seed bank. However, such strategies should be planned in the medium and long term and integrated with as many management practices as possible. Some difficulties can occur with this practice, such as the unevenness of hairy fleabane cycle at the application time since plants reach this stage at different times depending on emergence flows. Also, sensitivity variation among different glyphosate-resistant hairy fleabane populations is likely to occur.

Hairy fleabane plants can occur in various environments, not necessarily in crops. Glyphosate application (in total area or localized) at the early reproductive stage of hairy fleabane as a practice to reduce seed production and viability can be used in areas where there is no crop presence, such as native field areas, pastures, fallow areas, roadside borders, railroads, and boundaries between properties. Plants in these sites are often important sources for seed production, being dispersed toward many other places, including crop fields and urban areas. Another point to be carefully observed is that the increased number of glyphosate applications within productive systems increase selection pressure on weed populations of the same species and others.

The results of the present study indicated that glyphosate application affects hairy fleabane seed production. Glyphosate application at the vegetative stage of hairy fleabane reduced seed production, whereas, when applied at the early reproductive stage made seed production unfeasible. Glyphosate application had no effect on the viability of hairy fleabane seeds at the evaluated stages.

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